

# Abscisic Acid Activation of Plasma Membrane $\text{Ca}^{2+}$ Channels in Guard Cells Requires Cytosolic NAD(P)H and Is Differentially Disrupted Upstream and Downstream of Reactive Oxygen Species Production in *abi1-1* and *abi2-1* Protein Phosphatase 2C Mutants

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The hormone abscisic acid (ABA) regulates stress responses and developmental processes in plants. Calcium-permeable channels activated by reactive oxygen species (ROS) have been shown recently to function in the ABA signaling network in Arabidopsis guard cells. Here, we report that ABA activation of these  $\text{I}_{\text{Ca}}$   $\text{Ca}^{2+}$  channels requires the presence of NAD(P)H in the cytosol. The protein phosphatase 2C (PP2C) mutant *abi1-1* disrupted ABA activation of  $\text{I}_{\text{Ca}}$  channels. Moreover, in *abi1-1*, ABA did not induce ROS production. Consistent with these findings, in *abi1-1*,  $\text{H}_2\text{O}_2$  activation of  $\text{I}_{\text{Ca}}$  channels and  $\text{H}_2\text{O}_2$ -induced stomatal closing were not disrupted, suggesting that *abi1-1* impairs ABA signaling between ABA reception and ROS production. The *abi2-1* mutation, which lies in a distinct PP2C gene, also disrupted ABA activation of  $\text{I}_{\text{Ca}}$ . However, in contrast to *abi1-1*, *abi2-1* impaired both  $\text{H}_2\text{O}_2$  activation of  $\text{I}_{\text{Ca}}$  and  $\text{H}_2\text{O}_2$ -induced stomatal closing. Furthermore, ABA elicited ROS production in *abi2-1*. These data suggest a model with the following sequence of events in early ABA signal transduction: ABA, *abi1-1*, NAD(P)H-dependent ROS production, *abi2-1*,  $\text{I}_{\text{Ca}}$   $\text{Ca}^{2+}$  channel activation followed by stomatal closing.

## INTRODUCTION

The plant hormone abscisic acid (ABA) regulates a range of physiological processes, including seed maturation, control of vegetative growth, and promotion of dormancy, as well as tolerance of plants to adverse environmental conditions such as drought, cold, and salinity (Koorneef et al., 1998; Leung and Giraudat, 1998). In response to drought, ABA causes closing of stomatal pores, which are formed by pairs of guard cells in the epidermis of leaves and other aerial tissues. Stomatal closing results in a reduction of plant transpirational water loss. ABA induces an increase in cytosolic  $\text{Ca}^{2+}$  in guard cells, which precedes the reduction in sto-

matal aperture (McAinsh et al., 1990). Cytosolic  $\text{Ca}^{2+}$  elevation in turn activates slow (S-type) anion channels and downregulates inward  $\text{K}^+$  channels in guard cells (Schroeder and Hagiwara, 1989), resulting in net ion release and turgor reduction leading to stomatal closing. ABA increases  $\text{Ca}^{2+}$  by inducing both  $\text{Ca}^{2+}$  release from intracellular stores and  $\text{Ca}^{2+}$  influx from the extracellular space (Schroeder and Hagiwara, 1990; Grabov and Blatt, 1998; Leckie et al., 1998; Staxen et al., 1999; Hamilton et al., 2000; MacRobbie, 2000; Pei et al., 2000). ABA activation of plasma membrane  $\text{Ca}^{2+}$  influx also is required in Arabidopsis suspension culture cells, suggesting that ABA activation of  $\text{Ca}^{2+}$  influx is a more general component of ABA signaling in plants (Ghelis et al., 2000b). More than one type of plasma membrane  $\text{Ca}^{2+}$  channel may exist in guard cells (Schroeder and Hagiwara, 1990; Hamilton et al., 2000; Pei et al., 2000). The second messengers inositol 1,4,5-trisphosphate, cyclic ADP ribose, and calcium have been suggested to cause  $\text{Ca}^{2+}$  release via different endomembrane  $\text{Ca}^{2+}$  channels in response to ABA in guard cells (Gilroy et al., 1990; Ward and Schroeder, 1994; Parmar and Brearley, 1995; Lee et al., 1996; Leckie et al., 1998; Bewell et al., 1999; Staxen et al., 1999). Furthermore, a

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recent study also implicates inositol hexakisphosphate in  $\text{Ca}^{2+}$ -dependent stomatal movements (Lemtiri-Chlieh et al., 2000).

ABA triggers  $\text{Ca}^{2+}$  influx via nonselective  $\text{Ca}^{2+}$ -permeable channels in *Vicia* guard cells (Schroeder and Hagiwara, 1990). Cellular mechanisms that activate guard cell plasma membrane  $\text{Ca}^{2+}$  channels have been identified. Studies show that membrane hyperpolarization causes cytosolic  $\text{Ca}^{2+}$  increases in guard cells (Gilroy et al., 1991; Grabov and Blatt, 1998; Allen et al., 2000). Hyperpolarization-activated  $\text{Ca}^{2+}$  ( $I_{\text{Ca}}$ ) channels were identified in *Arabidopsis* and *Vicia* guard cells (Hamilton et al., 2000; Pei et al., 2000). In *Arabidopsis* the  $I_{\text{Ca}}$  currents were shown to be carried by non-selective cation channels (Pei et al., 2000). In *Vicia* guard cells, intracellular ABA transiently enhanced the activity of hyperpolarization-activated  $\text{Ca}^{2+}$  channels (Hamilton et al., 2000). In *Arabidopsis* guard cells,  $\text{H}_2\text{O}_2$  and ABA stimulated hyperpolarization-activated  $\text{Ca}^{2+}$ -permeable  $I_{\text{Ca}}$  channels (Pei et al., 2000).

ABA was shown to induce the production of reactive oxygen species (ROS) in *Arabidopsis* guard cells (Pei et al., 2000).  $\text{H}_2\text{O}_2$  activation of  $I_{\text{Ca}}$  channels and  $\text{H}_2\text{O}_2$ -induced stomatal closing were abolished in the ABA-insensitive mutant *gca2* (Himmelbach et al., 1998), providing genetic evidence for roles of ROS and  $I_{\text{Ca}}$  channels in ABA signaling (Pei et al., 2000). Interestingly, in maize embryos and *Vicia* guard cells, ABA was shown recently to increase  $\text{H}_2\text{O}_2$  levels (Guan et al., 2000; Zhang et al., 2001), indicating that ABA-induced ROS production may be of more general importance for ABA signaling. Diphenylene iodonium chloride (DPI), an inhibitor of NAD(P)H oxidases, partially inhibited ABA-induced stomatal closing (Pei et al., 2000). DPI also can inhibit other flavoenzymes (O'Donnell et al., 1993); therefore, further analyses are required to determine whether NAD(P)H contributes to the guard cell ABA response.

The dominant mutations *abi1-1* and *abi2-1* lie in two distinct type 2C protein phosphatases (PP2Cs) (Koorneef et al., 1984; Leung et al., 1994; Meyer et al., 1994; Grill and Himmelbach, 1998; Leung and Giraudat, 1998). The *abi1-1* and *abi2-1* mutations reduce ABA-induced cytosolic  $\text{Ca}^{2+}$  increases in guard cells (Allen et al., 1999a). Furthermore, experimentally imposing cytosolic  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) elevations bypasses these mutants and restores S-type anion channel activation and stomatal closing (Allen et al., 1999a), demonstrating that *abi1-1* and *abi2-1* disrupt early ABA signaling at the level of, or upstream of, ABA-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  increases. However, it remains unknown where these PP2C mutants act in the early signaling cascade and whether they affect ABA activation of  $I_{\text{Ca}}$  channels.

In this article, we investigate the link of ABA signaling to ROS production and  $I_{\text{Ca}}$  activation, and we analyze whether ABA activation of  $I_{\text{Ca}}$  depends on cytosolic NAD(P)H. Furthermore, we analyze whether *abi1-1* and *abi2-1* affect this newly recognized branch of ABA signaling, and if so, at what points in the signaling pathway. The results show a requirement of NAD(P)H in ABA activation of  $I_{\text{Ca}}$  and demon-

strate, via several independent analyses, that *abi1-1* and *abi2-1* differentially disrupt ABA activation of  $I_{\text{Ca}}$ .

## RESULTS

### NAD(P)H Requirement for ABA Activation of $\text{Ca}^{2+}$ Channels

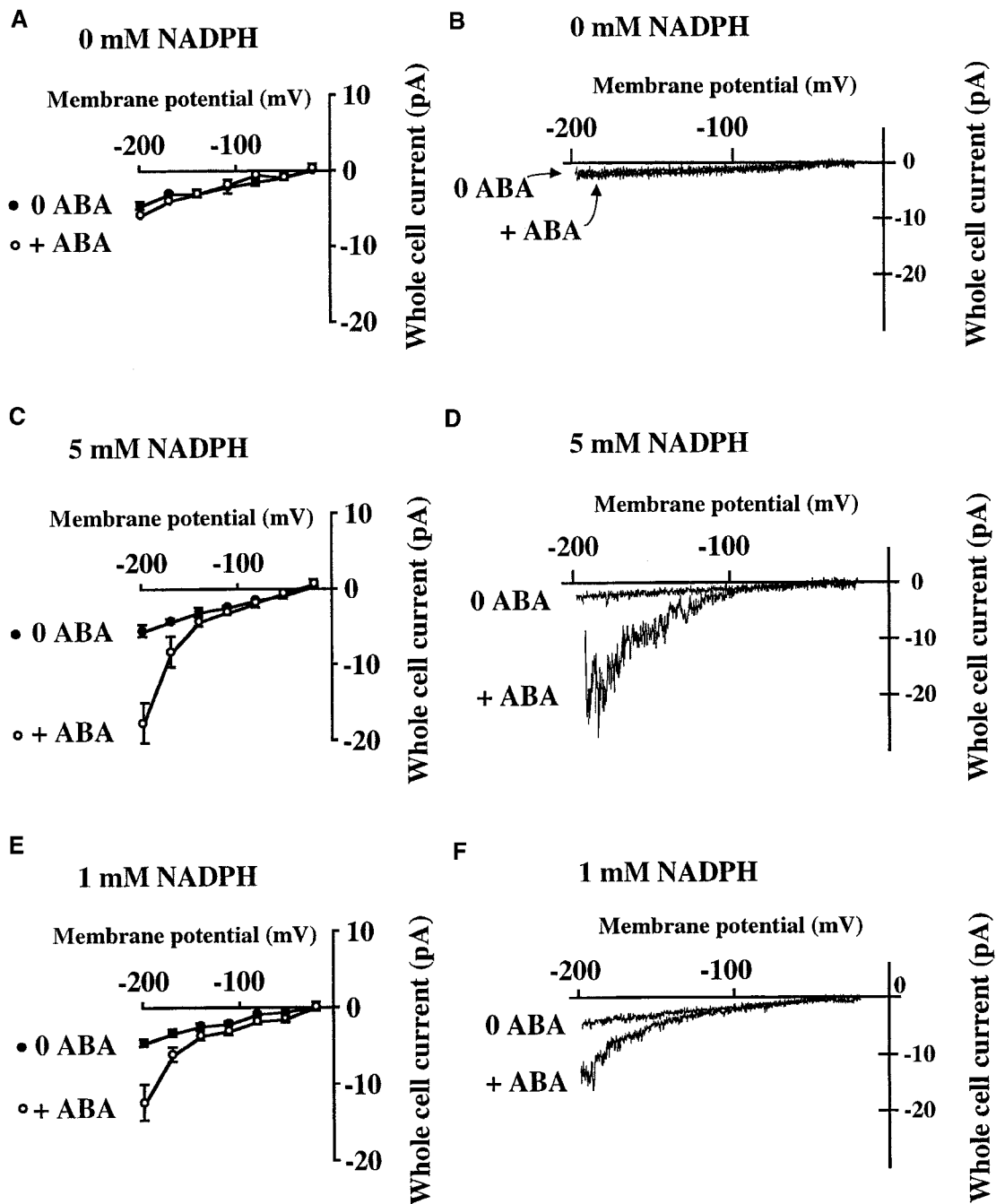
To examine whether NAD(P)H may play a role in the ABA stimulation of hyperpolarization-activated calcium-permeable channels, we analyzed ABA effects on  $I_{\text{Ca}}$  in the presence or absence of cytosolic NADPH. ABA and  $\text{H}_2\text{O}_2$  were applied to patch-clamped guard cells, and responses were recorded. The presence of 0.1 mM DTT in the patch clamp pipette and bath solutions inhibited the spontaneous activation of  $I_{\text{Ca}}$  currents, as reported previously (Pei et al., 2000). In the absence of DTT, some guard cells showed constitutive  $I_{\text{Ca}}$  activity (Pei et al., 2000), which supports the findings that oxidative processes activate these  $\text{Ca}^{2+}$  channels.

*Arabidopsis* guard cells (*Landsberg erecta* ecotype) were patch clamped for 10 min in the whole-cell mode before extracellular ABA application. When NADPH, the cytoplasm of guard cells, was not included in the patch clamp pipette solution which dialyzes, ABA did not activate  $I_{\text{Ca}}$  currents, as shown in Figures 1A and 1B ( $n = 10$ ). However, when 5 mM NADPH was added to the pipette solution, ABA activated  $I_{\text{Ca}}$  currents ( $n = 13$ ), as reported previously (Figures 1C and 1D) (Pei et al., 2000). Addition of the NADPH oxidase inhibitor DPI (12.5  $\mu\text{M}$ ) to the pipette solution inhibited the ABA activation of  $I_{\text{Ca}}$  ( $n = 3$ ; data not shown). ABA also activated  $I_{\text{Ca}}$  in the presence of 1 mM NADPH ( $P < 0.01$ ) (Figures 1E and 1F;  $n = 8$ ). The average amplitudes of ABA-activated whole-cell  $I_{\text{Ca}}$  currents at  $-198$  mV were statistically similar at 1  $\mu\text{M}$  and 5 mM NADPH ( $P > 0.11$ ).

In additional sets of experiments, 5 mM cytosolic NADPH caused an activation of  $I_{\text{Ca}}$  in the absence of added ABA in some cells, whereas with 1 mM cytosolic NADPH, ABA activation of  $I_{\text{Ca}}$  occurred (I.C. Mori, G.J. Allen, and J.I. Schroeder, data not shown). NADPH oxidation has been reported to be similar to NADH oxidation; therefore, not only NADPH but also NADH functions as a substrate of peroxidases to produce ROS in higher plants (Bestwick et al., 1998). When 5 mM NADPH was replaced with 5 mM NADH in the pipette solution, ABA activated  $I_{\text{Ca}}$  to a lesser extent, with an average amplitude of  $-7.8 \pm 1.6$  pA at  $-198$  mV ( $n = 9$ ; data not shown).

### *abi1-1* and *abi2-1* PP2C Mutants Disrupt the ABA Activation of $I_{\text{Ca}}$

To determine whether the *abi1-1* and *abi2-1* mutations affect the ABA activation of  $I_{\text{Ca}}$ , guard cell protoplasts of the *abi1-1* and *abi2-1* mutants were first patch clamped for  $\sim 10$  min in



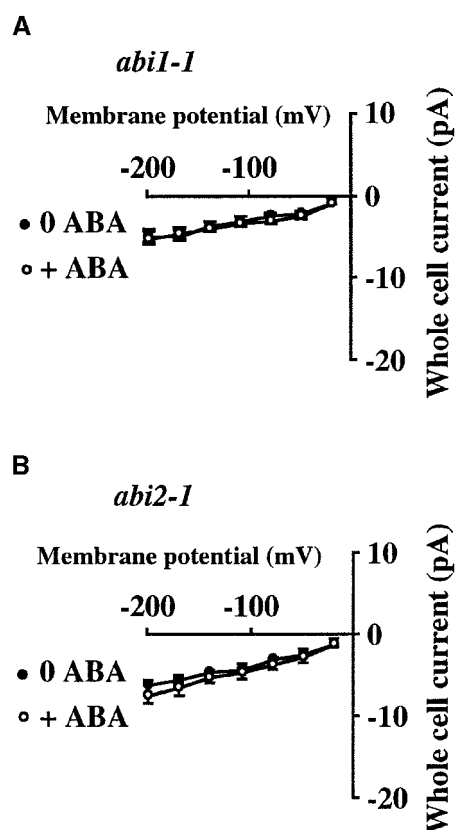
**Figure 1.** ABA Activation of Ca<sup>2+</sup>-Permeable I<sub>Ca</sub> Currents in Arabidopsis Guard Cell Protoplasts Requires Cytosolic NADPH.

(A) and (B) ABA (50  $\mu$ M) did not activate I<sub>Ca</sub> calcium channels when the pipette solution did not include NADPH or NADH. (B) shows two overlapping traces from a guard cell before and after ABA application.

(C) and (D) ABA (50  $\mu$ M) activated I<sub>Ca</sub> calcium currents when 5 mM NADPH was added to the pipette solution.

(E) and (F) ABA activated I<sub>Ca</sub> when 1 mM NADPH was added to the pipette solution.

(A), (C), and (E) show average responses, and (B), (D), and (F) show responses in individual cells before and 5 min after ABA application. ABA was added  $\sim$ 10 min after establishing whole-cell recordings, and whole-cell currents were measured before ABA application and in the same cells 5 min after extracellular ABA application in all recordings. The numbers of cells averaged are given in the text. Closed circles, before ABA addition to batch solution; open circles, 5 min after ABA addition to the same cells. Error bars represent SEM.



**Figure 2.** ABA Failed to Activate  $\text{Ca}^{2+}$  Channel Currents in *abi1-1* and *abi2-1* PP2C Mutant Guard Cells.

**(A)** ABA (50  $\mu\text{M}$ ) did not activate  $I_{\text{Ca}}$  in *abi1-1* guard cells with 5 mM NADPH included in the pipette solution ( $n = 5$ ).

**(B)** ABA (50  $\mu\text{M}$ ) did not activate  $I_{\text{Ca}}$  in *abi2-1* guard cells with 5 mM NADPH added to the pipette solution ( $n = 5$ ).

Experiments were performed as described in Figure 1. Error bars represent SEM.

the whole-cell mode. Subsequently, the same cells were treated with ABA by bath perfusion. Figure 2 shows the effects of *abi1-1* and *abi2-1* on the ABA activation of  $I_{\text{Ca}}$ . ABA did not activate  $I_{\text{Ca}}$  in either mutant in the presence of 5 mM cytosolic NADPH ( $n = 5$ ,  $P > 0.72$  for *abi1-1*;  $n = 5$ ,  $P > 0.35$  for *abi2-1*).

#### **$\text{H}_2\text{O}_2$ -Induced Responses Are Impaired in *abi2-1* but Not in *abi1-1***

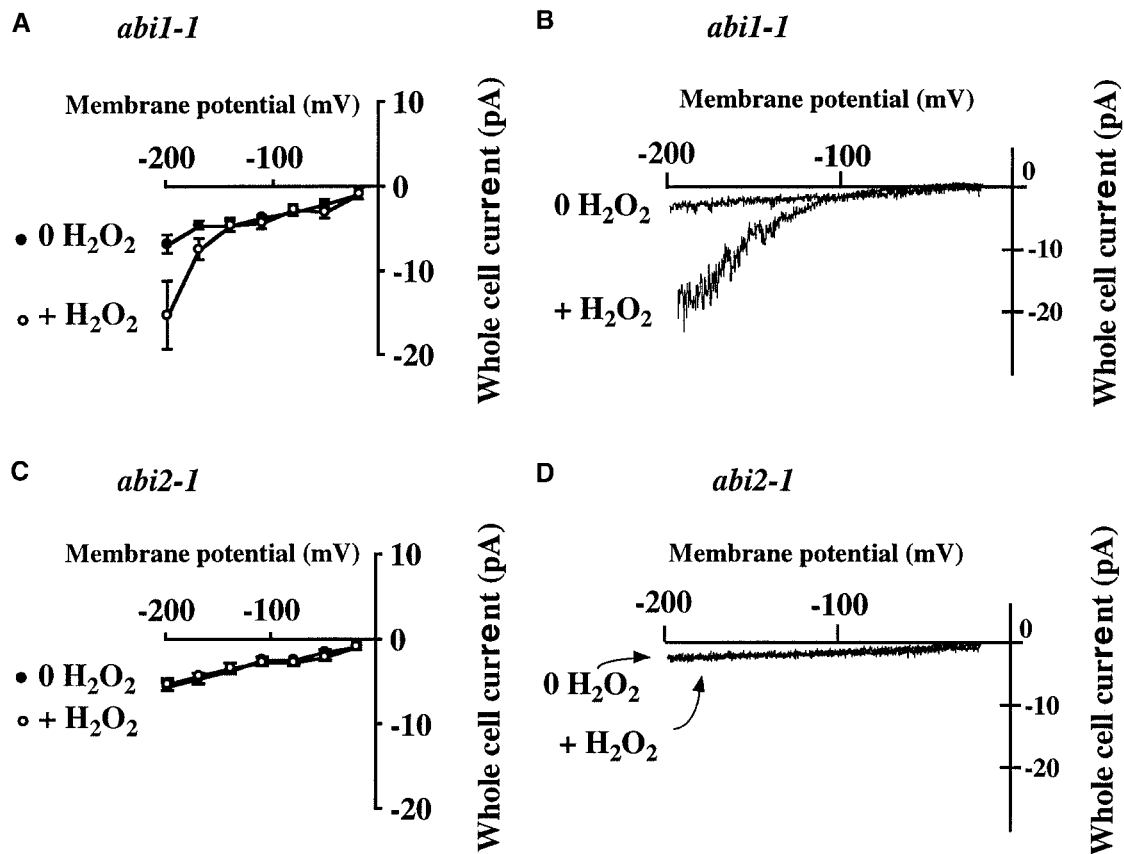
To examine whether the *abi1-1* and *abi2-1* mutations impair the activation of  $I_{\text{Ca}}$  upstream or downstream of or parallel to ROS production, we first analyzed  $\text{H}_2\text{O}_2$  activation of the hy-

perpolarization-activated  $\text{Ca}^{2+}$  channel currents in *abi1-1* and *abi2-1*.  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ) clearly activated  $I_{\text{Ca}}$  in *abi1-1* guard cells, as shown in Figures 3A and 3B ( $n = 6$ ,  $P < 0.04$ ). Interestingly, however,  $\text{H}_2\text{O}_2$  failed to activate  $I_{\text{Ca}}$  in *abi2-1* guard cells (Figures 3C and 3D) ( $n = 5$ ,  $P > 0.84$ ).

Exposure of stomates to  $\text{H}_2\text{O}_2$  induces  $[\text{Ca}^{2+}]_{\text{cyt}}$  increases and partial stomatal closing in *Commelina* and *Arabidopsis* (McAinsh et al., 1996; Pei et al., 2000). To further analyze  $\text{H}_2\text{O}_2$ -mediated signal transduction in *abi1-1* and *abi2-1*, we performed stomatal closing assays in wild-type, *abi1-1*, and *abi2-1* leaves. As reported previously, extracellular  $\text{Ca}^{2+}$  is required for  $\text{H}_2\text{O}_2$  induction of stomatal closing and  $[\text{Ca}^{2+}]_{\text{cyt}}$  increases (Pei et al., 2000). When 0.1 mM  $\text{CaCl}_2$  was added to the bath solution, partial stomatal closing occurred (DeSilva et al., 1985; Allen et al., 1999a). However, buffering the total free  $\text{Ca}^{2+}$  concentration to  $\sim 0.1$  mM in the cell wall space of epidermal strips with a bath solution containing 0.1 mM EGTA and 0.2 mM  $\text{Ca}^{2+}$  minimized  $\text{Ca}^{2+}$ -induced stomatal closing and allowed analysis of  $\text{H}_2\text{O}_2$  responses (Pei et al., 2000).  $\text{H}_2\text{O}_2$  at 100  $\mu\text{M}$  triggered a reduction in stomatal aperture in the wild type (*Landsberg erecta* ecotype), as illustrated in Figure 4A ( $n = 3$  experiments,  $P < 0.01$ ). The partial  $\text{H}_2\text{O}_2$  response compared with the ABA response (Figure 4A) is consistent with the proposed model of parallel branches together mediating early ABA signaling (see Figure 5f in the article by Pei et al., 2000).  $\text{H}_2\text{O}_2$  also triggered a reduction in stomatal aperture in the *abi1-1* mutant (Figure 4B;  $n = 3$ ,  $P < 0.02$ ). However, stomatal aperture measurements showed that  $\text{H}_2\text{O}_2$ -induced stomatal closing was impaired in the *abi2-1* mutant (Figure 4C;  $n = 3$ ,  $P > 0.46$ ). Stomatal movement results were confirmed in additional control and blind experiments (see Methods).

#### **ABA Enhances ROS Levels in the Wild Type and *abi2-1* but Not in *abi1-1***

To analyze ABA-dependent ROS production in *abi1-1* and *abi2-1*, ROS levels were analyzed in populations of wild-type, *abi1-1*, and *abi2-1* guard cells using the fluorescent dye 2',7'-dichlorofluorescein diacetate ( $\text{H}_2\text{DCF-DA}$ ), which reports changes in the oxidative state of guard cells (Ohba et al., 1994; Lee et al., 1999; Pei et al., 2000). As shown in Figure 5, the relative fluorescence emission increased after treatment of wild-type guard cells with 50  $\mu\text{M}$  ABA (four experiments,  $P < 0.02$ ). However, ROS measurements showed that 50  $\mu\text{M}$  ABA did not increase the relative fluorescence emission in *abi1-1* guard cells (six experiments). A slight ABA-induced decrease in ROS levels was observed in *abi1-1* guard cells, which was not significant in all data sets ( $P = 0.02$  to  $0.053$ ) (Figure 5). Conversely, ABA at 50  $\mu\text{M}$  increased the relative fluorescence emission in *abi2-1* guard cells (nine experiments,  $P < 0.001$ ) (Figure 5). Impairment of ABA-induced ROS production in *abi1-1* was significant compared with that in the wild type ( $P < 0.001$ ) and *abi2-1* ( $P < 0.001$ ). Differential ABA-induced fluorescence responses in



**Figure 3.** H<sub>2</sub>O<sub>2</sub> Activates Ca<sup>2+</sup>-Permeable Currents in *abi1-1* but Not *abi2-1* Arabidopsis Guard Cell Protoplasts.

(A) and (B) H<sub>2</sub>O<sub>2</sub> (100 μM) activates I<sub>Ca</sub> in *abi1-1* guard cells (*n* = 6).

(C) and (D) H<sub>2</sub>O<sub>2</sub> (100 μM) did not activate I<sub>Ca</sub> in *abi2-1* guard cells (*n* = 5). (D) shows two overlapping traces.

Experiments were performed as described in Figure 1 except that H<sub>2</sub>O<sub>2</sub> was used instead of ABA. Error bars represent SEM.

*abi1-1* and *abi2-1* were confirmed in additional blind experiments (see Methods).

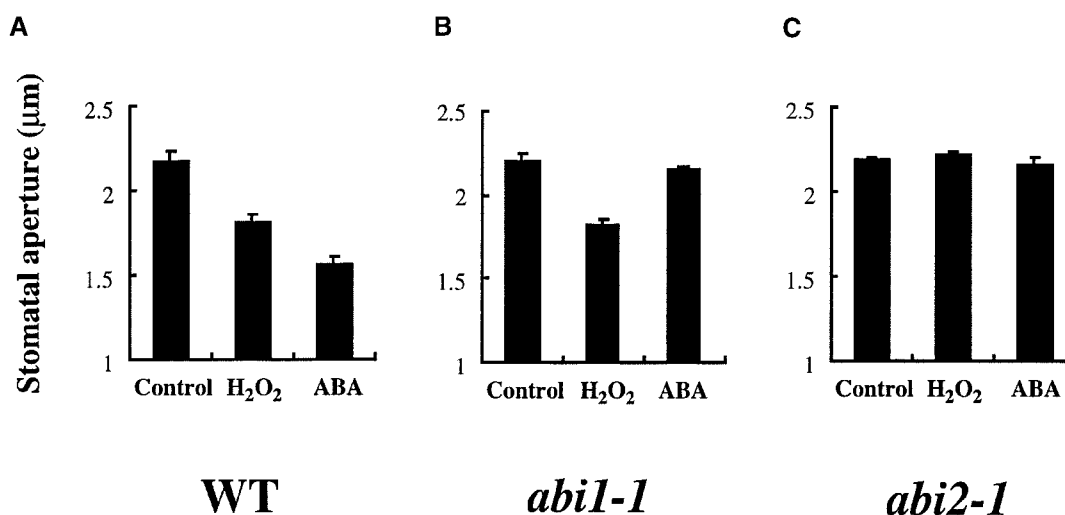
## DISCUSSION

ABA induces an increase in [Ca<sup>2+</sup>]<sub>cyt</sub>, leading to a reduction in stomatal aperture. Recent studies of Arabidopsis mesophyll suspension culture cells showed that ABA induction of *Rab18* gene expression requires ABA activation of plasma membrane Ca<sup>2+</sup> influx followed by S-type anion channel activation, suggesting that the analyzed early signaling mechanisms in guard cells may be components of ABA signaling in many plant cell types (Ghelis et al., 2000a, 2000b). ABA stimulates hyperpolarization-activated I<sub>Ca</sub> Ca<sup>2+</sup> channels via ROS production, suggesting a new branch in early ABA signaling (Pei et al., 2000). ABA also increases the endogenous

level of ROS in maize embryos, suggesting that H<sub>2</sub>O<sub>2</sub> functions in ABA regulation of seed maturation (Guan et al., 2000). Furthermore, recent studies have linked ABA with oxidative responses (Bueno et al., 1998; Gong et al., 1998). These studies suggest that ROS may be of more general importance for ABA signal transduction in plants.

### Cytosolic NADPH Is Necessary for the ABA Activation of I<sub>Ca</sub>

ROS is a term for radicals and other reactive species derived from oxygen. ROS have been implicated in numerous signal transduction pathways in both plant and animal cells (Lamb and Dixon, 1997; Rhee et al., 2000). In plants, ROS, including the superoxide radical and H<sub>2</sub>O<sub>2</sub>, act as important second messengers in defense responses triggered by pathogens and elicitors (Levine et al., 1994; Lamb and Dixon,



**Figure 4.** Differential Effects of H<sub>2</sub>O<sub>2</sub> on Stomatal Apertures of *abi1-1* and *abi2-1*.

(A) Both ABA (50 µM) and H<sub>2</sub>O<sub>2</sub> (100 µM) induced stomatal closing in the wild type (*Landsberg erecta* ecotype).

(B) ABA did not cause stomatal closing, but H<sub>2</sub>O<sub>2</sub> elicited partial stomatal closing in *abi1-1*.

(C) Neither ABA nor H<sub>2</sub>O<sub>2</sub> elicited stomatal closing of *abi2-1*.

Averages from  $n =$  three leaf epidermal experiments are shown (60 stomates per bar). Error bars represent SEM.

1997). Many enzymes can produce ROS in plant cells. Activation of these enzymes is closely associated with oxidative bursts (Lamb and Dixon, 1997). A plasma membrane oxidase generates superoxide (Keller et al., 1998), a peroxidase produces H<sub>2</sub>O<sub>2</sub> directly, and an oxalate oxidase also generates H<sub>2</sub>O<sub>2</sub> (Baker and Orlandi, 1995). DPI, an inhibitor of neutrophil NADPH oxidases (Cross and Jones, 1986, 1991), partially inhibits ABA-induced stomatal closing (Pei et al., 2000). The elicitors oligogalacturonic acid and chitosan reduce stomatal aperture and induce the production of ROS in tomato and *Commelina* guard cells (Lee et al., 1999). The ROS activation of I<sub>Ca</sub> channels has been proposed to represent a possible joint branch of multiple stress signaling pathways (Pei et al., 2000; Schroeder et al., 2001).

Previous studies have shown that the ABA activation of Ca<sup>2+</sup> channels in *Vicia* guard cells is transient and attenuated (Schroeder and Hagiwara, 1990; Hamilton et al., 2000). In the present study of *Arabidopsis* guard cells, we obtained ABA activation of I<sub>Ca</sub> in patch-clamped whole cells by adding NADPH or NADH via the patch pipette to the cytosol of guard cells. However, no ABA response was found in the relatively small *Arabidopsis* guard cells when no NAD(P)H was added to the patch pipette. The requirement of NADPH or NADH, ABA-induced ROS production, and the inhibitory effects of DPI suggest that NAD(P)H oxidases and/or redox control of sulfhydryl groups contributes to ABA signal transduction. NAD(P)H is formed by the reduction of NAD(P) via light-supplied energy in guard cell chloroplasts (Shimazaki et al., 1989). A previous study showed that ABA-induced

stomatal closing requires guard cell metabolism (Weyers et al., 1982). In this respect, the results presented here indicate a possible link between guard cell metabolism and ion channel regulation. ABA signaling requires hydrolyzable ATP in guard cells (Schmidt et al., 1995) and phosphorylation events as positive transducers of stomatal closing, which also would require intact guard cell metabolism.

#### Plasma Membrane Ca<sup>2+</sup> Channel Activity in Guard Cells

Guard cells show spontaneous activity of hyperpolarization-induced Ca<sup>2+</sup> increases (Gilroy et al., 1991; Grabov and Blatt, 1998; Allen et al., 1999b) and spontaneous activity of plasma membrane Ca<sup>2+</sup> currents (Hamilton et al., 2000; Pei et al., 2000). Whether the spontaneous Ca<sup>2+</sup> influx and I<sub>Ca</sub> are mediated by the same Ca<sup>2+</sup> channel remains unknown. Interestingly, the spontaneous activity of hyperpolarization-induced Ca<sup>2+</sup> currents was inhibited by adding DTT to the patch clamp pipette (cytosolic) solution and bath solutions (Pei et al., 2000; this study).

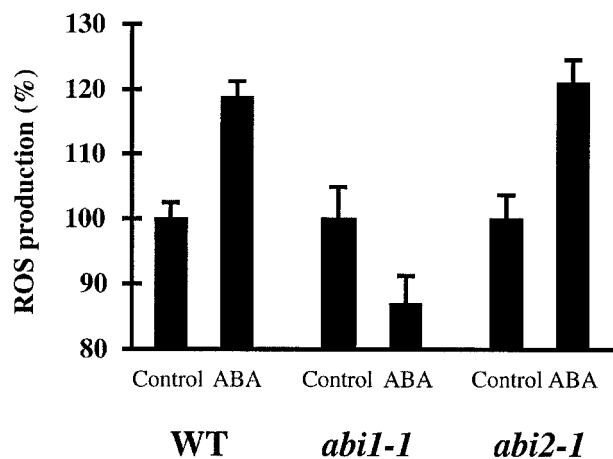
Fungal elicitors have been shown to induce hyperpolarization-activated Ca<sup>2+</sup> channels in tomato suspension culture cells (Gelli et al., 1997). These Ca<sup>2+</sup> channels show a similar I<sub>Ca</sub>-like activation by hyperpolarization and a more pronounced time-dependent activation. Interestingly, in some tomato cells, spontaneous activity of hyperpolarization-activated Ca<sup>2+</sup> channels was observed, which also was inhibited by 1 mM DTT (A. Gelli and E. Blumwald, personal

communication). Pathogenic elicitors cause ROS production in plants, with Ca<sup>2+</sup> influx occurring both before and after ROS production (Knight et al., 1991; Price et al., 1994; Lamb and Dixon, 1997; Kawano et al., 1998), suggesting that more than one Ca<sup>2+</sup> channel or activation mechanism may contribute to this response. I<sub>Ca</sub>-like Ca<sup>2+</sup> channels may contribute to the secondary response that follows ROS production.

Interestingly, hyperpolarization-activated Ca<sup>2+</sup> channels also have been identified in Arabidopsis cells from the cortical elongation zone of roots and the epidermis of the growing root tip, but not in mature epidermis or in pericycle cells (Kiegle et al., 2000) or in the apex of Arabidopsis root hair cells (Very and Davies, 2000). These studies suggest that hyperpolarization-activated Ca<sup>2+</sup> channels may contribute to various signal transduction and growth processes in plants, and their opposite voltage dependence compared with depolarization-activated Ca<sup>2+</sup> channels (Huang et al., 1994; Marshall et al., 1994; Thuleau et al., 1994) suggests activation during different signaling processes.

#### Differential Disruption by *abi1-1* and *abi2-1* PP2Cs

Stomata of the *abi1-1* and *abi2-1* mutants are insensitive to ABA (Finkelstein and Somerville, 1990; Roelfsema and Prins,



**Figure 5.** Differential Production of ROS in *abi1-1* and *abi2-1* Guard Cells Treated with ABA.

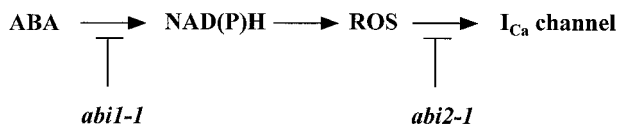
ABA (50  $\mu$ M) increased ROS production in the wild type (WT) (four experiments,  $n = 161$  cells before ABA treatment,  $n = 123$  cells after ABA treatment) and *abi2-1* (nine experiments,  $n = 221$  cells before ABA treatment,  $n = 207$  cells after ABA treatment). ABA did not increase ROS production in *abi1-1* (six experiments,  $n = 110$  cells before ABA treatment,  $n = 150$  cells after ABA treatment). Changes in ROS levels were analyzed by measuring H<sub>2</sub>DCF fluorescence levels in guard cells in response to ABA or solvent (0.1% ethanol) control applications. Error bars represent SEM.

1995; Pei et al., 1997). ABA regulation of K<sup>+</sup> channels is impaired by *abi1-1* expression (Armstrong et al., 1995), and ABA activation of S-type anion channels is impaired in *abi1-1* and *abi2-1* (Pei et al., 1997). The Arabidopsis *abi1-1* and *abi2-1* mutations impair ABA-induced cytoplasmic Ca<sup>2+</sup> increases in guard cells (Allen et al., 1999a). ABA induces both Ca<sup>2+</sup> release from intracellular stores and Ca<sup>2+</sup> influx from the extracellular space. However, it remained unknown which Ca<sup>2+</sup> increase mechanisms were affected by *abi1-1* and *abi2-1*. The identification of ROS and I<sub>Ca</sub> as early ABA signaling intermediates has allowed a direct analysis of the effects of the *abi* PP2C mutations on early signal transduction mechanisms.

Here, we show that the PP2C mutations *abi1-1* and *abi2-1* both disrupt the ABA activation of I<sub>Ca</sub>. ABA did not induce the production of ROS in the *abi1-1* mutant. H<sub>2</sub>O<sub>2</sub>-activated I<sub>Ca</sub> and H<sub>2</sub>O<sub>2</sub>-induced stomatal closing were not impaired in *abi1-1*. These findings suggest that the *abi1-1* mutation disrupts ABA signaling upstream of ROS production, as illustrated in Figure 6. In contrast, ABA elicited ROS production in the *abi2-1* mutant, but H<sub>2</sub>O<sub>2</sub> did not activate I<sub>Ca</sub> and did not induce stomatal closing in *abi2-1*. These data suggest that the *abi2-1* mutation impairs ABA signaling downstream of ROS production (Figure 6). These data lead to a simple model for the positioning of I<sub>Ca</sub>, ROS production, and the *abi1-1* and *abi2-1* protein phosphatases in the ABA signal transduction cascade in Arabidopsis guard cells, as illustrated in Figure 6. Note that ABA may function by downregulating ROS-scavenging enzymes such as catalase. The data further suggest that *abi1-1* interacts with early signal transduction mechanisms upstream of ROS production. Previous studies have suggested that *abi1-1* and *abi2-1* have distinct functions, even though they both disrupt ABA signaling in general (Gilmour and Thomashow, 1991; Vartanian et al., 1994; Gosti et al., 1995; Bruxelles et al., 1996; Söderman et al., 1996; Pei et al., 1997; Strizhov et al., 1997). Our data are consistent with these findings and provide a working model for the ABA signal transduction pathway to explain the differential effects of *abi1-1* and *abi2-1* (Figure 6).

It remains unknown whether *abi1-1* and *abi2-1* additionally affect Ca<sup>2+</sup> release from intracellular stores and a possible parallel Ca<sup>2+</sup>-independent pathway (Allan et al., 1994; Allen et al., 1999a). Ca<sup>2+</sup> release can occur parallel to Ca<sup>2+</sup> influx at low ABA concentrations (MacRobbie, 2000), and perhaps also downstream of I<sub>Ca</sub> activation, based on Ca<sup>2+</sup> activation of plant phospholipase C isoforms (Staxen et al., 1999) and on the proposed Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release via vacuolar slow vacuolar (SV) channels (Ward and Schroeder, 1994; Bewell et al., 1999). If *abi1-1* impairs an ABA receptor, parallel mechanisms could be accounted for by one effect of *abi1-1*. Note that the *abi1-1* and *abi2-1* PP2Cs may interact with more than one protein. Future research is needed to analyze the effects of *abi1-1* and *abi2-1* on Ca<sup>2+</sup> release mechanisms.

In summary, the present study provides strong support for the new model that ABA-induced ROS production and



**Figure 6.** Model Summarizing the Differential Disruption of ABA Activation of  $I_{Ca}$   $Ca^{2+}$  Channels by the Two *abi1-1* and *abi2-1* PP2C Mutants.

ABA activation of  $Ca^{2+}$  channels required cytosolic NAD(P)H, was inhibited by DPI, and was accompanied by ROS production.

$I_{Ca}$   $Ca^{2+}$ -permeable channel activation are important components of ABA signal transduction. The cytosolic NAD(P)H requirement for ABA activation of  $I_{Ca}$  channels, together with DTT and DPI inhibition of  $I_{Ca}$ , suggests that NAD(P)H oxidases and/or redox control of sulfhydryl groups contributes to early ABA signaling. Furthermore, *abi1-1* and *abi2-1* interact with important and distinct transducers of this early ABA signaling branch upstream and downstream of ROS production, respectively.

## METHODS

### Isolation of Arabidopsis Guard Cells

*Arabidopsis thaliana* wild type (Landsberg *erecta* ecotype) and the *abi1-1* and *abi2-1* mutant lines were used in this study. The mutant genotypes and homozygosity were confirmed using a previously described polymerase chain reaction method (Leung et al., 1997). The abscisic acid (ABA) insensitivity was further confirmed in seed germination assays (Pei et al., 1997; Allen et al., 1999a). Note that during the course of this work, a separate set of *abi1-1* and *abi2-1* seed was obtained from the Arabidopsis stock center (Ohio State University, Columbus). However, seed germination and polymerase chain reaction tests showed that these consisted of a mixed population. The stock center was informed, and these seed were not used further. Plants were grown in soil in plant growth chambers with a 16-hr-light (80  $\mu$ E light fluence rate) and 8-hr-dark regimen and watered with deionized water every day. Arabidopsis guard cell protoplasts were isolated enzymatically from leaf epidermal strips of 4- to 6-week-old plants. Arabidopsis rosette leaves were blended in a commercial blender in deionized water three times for 5 sec each and collected using a nylon mesh (pore size, 62  $\mu$ m). The collected epidermal tissue was incubated in 10 mL of medium containing 1% Cellulase R-10, 0.5% Macerozyme R-10 (Yakult, Japan), 0.5% BSA, 0.5 M mannitol, 0.1 mM KCl, 0.1 mM  $CaCl_2$ , 10 mM ascorbic acid, and 0.1% kanamycin sulfate, pH 5.5 (with KOH), for 15 to 17 hr at 24°C on a shaker. Isolated guard cell protoplasts were collected and washed twice as described previously (Pei et al., 1997).

### Patch Clamp and Data Acquisition

Whole-cell patch clamp recordings from Arabidopsis guard cells were made using Axopatch 200 and 200A amplifiers (Axon Instru-

ments, Union City, CA) that were connected to microcomputers via interfaces as described (Pei et al., 1997). Seal resistances were  $>10$  G $\Omega$ . Liquid junction potentials were corrected (Ward and Schroeder, 1994). Initially, upon establishment of whole-cell recordings, a current was observed in some guard cells. This current, however, disappeared within 30 sec to 4 min after establishing whole-cell recordings in 90% of the guard cells analyzed. The frequency of occurrence of this initial current varied from cell preparation to cell preparation. After the initial current had vanished and  $\sim 10$  min after establishing whole-cell recordings, ABA and  $H_2O_2$  were applied by bath perfusion to patch-clamped guard cells and responses were recorded. pClamp software (Axon Instruments) was used to acquire and analyze whole-cell currents. The standard voltage protocol ramped from  $-18$  to  $-198$  mV (ramp speed, 180 mV/sec). The interpulse period was 1 min. Whole-cell currents were not leak subtracted. Data were analyzed using Axograph software (Axon Instruments, Inc., Foster City, CA). The bath solution used in patch clamp experiments contained 100 mM  $BaCl_2$ , 0.1 mM DTT, and 10 mM Mes titrated to pH 5.6 with Tris, and the pipette solution was composed of 10 mM  $BaCl_2$ , 0.1 mM DTT, 4 mM EGTA, 10 mM Hepes adjusted to pH 7.1, and Tris. To investigate the effects of ABA on a  $Ca^{2+}$ -permeable, non-selective cation current ( $I_{Ca}$ ), NADPH or NADH was added to the pipette solution at the indicated concentrations in the text. To analyze the hyperpolarization-activated currents, we used  $Ba^{2+}$  ions, which are permeable to  $I_{Ca}$  channels (Pei et al., 2000).

### Stomatal Aperture Measurements

Stomatal movement analyses were performed as described previously (Pei et al., 2000). Rosette leaves from 4- to 6-week-old plants were exposed to white light (125  $\mu$ E fluence rate) while floating in a solution containing 10 mM KCl, 0.2 mM  $CaCl_2$ , 0.1 mM EGTA (free extracellular  $Ca^{2+}$  concentration buffered to  $\sim 0.1$  mM), and 10 mM Mes titrated to pH 6.15 with KOH. Subsequently, 100  $\mu$ M  $H_2O_2$  and 50  $\mu$ M ABA were added to the bath solution as indicated in the Figures and text. After treatment for 2 hr in white light (125  $\mu$ E fluence rate), leaves were blotted and stomatal apertures were measured by focusing on the inner lips of stomates (away from the focal plane of guard cells) as described (Ichida et al., 1997). In each epidermal peel experiment, 20 stomatal apertures were measured at each condition. In additional control and blind experiments, the ability of  $H_2O_2$  to cause stomatal closing in wild-type and *abi1-1* leaves, but not *abi2-1* leaves, was reproduced ( $n = 3$  experiments per line; wild type  $\pm H_2O_2$ ,  $P < 0.03$ ; *abi1-1*  $\pm H_2O_2$ ,  $P < 0.02$ ; *abi2-1*  $\pm H_2O_2$ ,  $P > 0.36$ ). Standard errors were determined relative to the square root of the number of epidermal strip experiments, as in previous studies (Ichida et al., 1997; Pei et al., 2000). All statistical analyses were performed using the TTEST program in Excel 5.0 software (Microsoft, Redmond, WA). Values of  $P < 0.05$  were considered to show statistically significant differences.

### Reactive Oxygen Species Detection in Guard Cells

Reactive oxygen species (ROS) production in guard cells was analyzed using 2',7'-dichlorofluorescein diacetate ( $H_2DCF$ -DA) (Ohba et al., 1994; Lee et al., 1999). This nonfluorescent compound is permeable to the plasma membrane and is converted to dichlorofluorescein ( $H_2DCF$ ), which is impermeable.  $H_2DCF$  can be oxidized by peroxi-



dases and H<sub>2</sub>O<sub>2</sub>. Epidermal tissues were isolated from 6-week-old plants with a commercial blender. The epidermal tissues were incubated in 30 mM KCl and 10 mM Mes-KOH, pH 6.15, in the light at room temperature for 2 hr (Pei et al., 2000). Note that immediately after epidermal tissue isolations, guard cells showed increased ROS levels, likely as a result of mechanical perturbation from epidermis excision. However, after 2-hr incubations of epidermal tissues in white light (125 μE), ROS levels decreased. Fifty micromolar H<sub>2</sub>DCF-DA was added to the incubation medium and then either 0.1% ethanol (control) or 50 μM ABA was added to the incubation medium after 20 to 30 min of dye loading. The epidermal tissues were collected using a nylon mesh and washed with distilled water twice after 20 to 30 min of ABA or ethanol control treatments. Guard cells were observed under a fluorescence microscope equipped with a cooled charge-coupled device camera. Note that prolonged exposure of H<sub>2</sub>DCF-loaded guard cells to excitation light led to a transient increase in ROS and subsequent bleaching of the dye. Therefore, to compare fluorescence responses in control and ABA-treated samples, excitation light exposure was reduced using neutral density filters and limited to a 10-sec exposure, and only one image was captured per sample. All experiments reported here (Figure 5) were confirmed in additional blind experiments (*abi2-1* ± ABA, five experiments, *n* = 234 guard cells, *P* < 0.01). *abi1-1* showed a slight but statistically insignificant ABA-induced reduction in ROS (five experiments, *n* = 152 guard cells, *P* = 0.053). Images were acquired and the fluorescence emission of guard cells was analyzed using Adobe Photoshop 5.0 (Mountain View, CA).

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## Correction

**Yoshiyuki Murata, Zhen-Ming Pei, Izumi C. Mori, and Julian Schroeder.** (2001). Abscisic Acid Activation of Plasma Membrane  $\text{Ca}^{2+}$  Channels in Guard Cells Requires Cytosolic NAD(P)H and Is Differentially Disrupted Upstream and Downstream of Reactive Oxygen Species Production in *abi1-1* and *abi2-1* Protein Phosphatase 2C Mutants. *Plant Cell* **13**, 2513–2523.

On page 2514 (**RESULTS**) under the heading “**NAD(P)H Requirement for ABA Activation of  $\text{Ca}^{2+}$  Channels**” in the second paragraph, two sentences were erroneously edited and should have appeared as:

“When NAD(P)H was not included in the patch clamp pipette solution, which dialyzes the cytoplasm of guard cells, ABA did not activate  $I_{\text{Ca}}$  currents, as shown in Figures 1A and 1B ( $n = 10$ ).”

“The average amplitudes of ABA-activated whole-cell  $I_{\text{Ca}}$  currents at  $-198$  mV were statistically similar at 1 mM and 5 mM NAD(P)H ( $P > 0.11$ ).”

**Abscisic Acid Activation of Plasma Membrane Ca<sup>2+</sup> Channels in Guard Cells Requires Cytosolic NAD(P)H and Is Differentially Disrupted Upstream and Downstream of Reactive Oxygen Species Production in *abi1-1* and *abi2-1* Protein Phosphatase 2C Mutants**

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